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Autoregulation of *Sinorhizobium meliloti exoR* gene expression

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The successful nitrogen-fixing symbiosis between the Gram-negative soil bacterium *Sinorhizobium meliloti* and its leguminous plant host alfalfa (*Medicago sativa*) requires the bacterial exopolysaccharide succinoglycan. Succinoglycan and flagellum production, along with the ability to metabolize more than 20 different carbon sources and control the expression of a large number of *S. meliloti* genes, is regulated by the ExoR–ExoS/ChvI signalling pathway. The ExoR protein interacts with and suppresses the sensing activities of ExoS, the membrane-bound sensor of the ExoS/ChvI two-component regulatory system. Here we show that *exoR* expression is clearly upregulated in the absence of any functional ExoR protein. This upregulation was suppressed by the presence of the wild-type ExoR protein but not by a mutated ExoR protein lacking signal peptide. The levels of *exoR* expression could be directly modified in real-time by changing the levels of total ExoR protein. The expression of *exoR* was also upregulated by the constitutively active sensor mutation *exoS96*, and blocked by two single mutations, *exoS** and $exoS_{supA}$, in the ExoS sensing domain. Presence of the wild-type ExoS protein further elevated the levels of *exoR* expression in the absence of functional ExoR protein, and reversed the effects of *exoS96*, *exoS** and $exoS_{supA}$ mutations. Altogether, these data suggest that ExoR protein autoregulates *exoR* expression through the ExoS/ChvI system, allowing *S. meliloti* cells to maintain the levels of *exoR* expression based on the amount of total ExoR protein.

INTRODUCTION

Bacterial sensing plays an essential role in the establishment of nitrogen-fixing symbiosis between the Gram-negative soil bacterium *Sinorhizobium meliloti* and its leguminous plant partner alfalfa (*Medicago sativa*) (Gage, 2004; Jones *et al.*, 2007). The sensing of plant signalling molecules – flavonoids – leads to the production of nodulation factor by *S. meliloti*, which induces the formation of curled root hairs that are colonized by *S. meliloti* cells (Brewin, 1991; Gibson *et al.*, 2008; Long, 1989, 2001; van Rhijn & Vanderleyden, 1995). The success of the next step of the symbiosis, the formation of infection threads in the colonized curled alfalfa root hairs, requires the presence of the *S. meliloti* exopolysaccharide succinoglycan, which is regulated by the *S. meliloti* ExoR–ExoS/ChvI signal-transduction pathway (Cheng & Walker, 1998a, b; Doherty *et al.*, 1988; Krol & Becker, 2009; Leigh & Walker, 1994; Pellock *et al.*, 2000). These infection threads serve as the entryway for *S. meliloti* cells to colonize and establish nitrogen-fixing symbiosis inside pink alfalfa root nodules (Gibson *et al.*, 2008; Jones *et al.*, 2007).

Abbreviation: CF, calcofluor white.

The *S. meliloti exoR* gene, encoding a 268-amino-acid ExoR protein, was initially discovered with the isolation of the *exoR95*::Tn*5* mutant (Doherty *et al.*, 1988; Reed *et al.*, 1991). This loss-of-function mutant overproduces succinoglycan and terminates the production of flagella by modulating the expression of biosynthesis genes (Wells *et al.*, 2007; Yao *et al.*, 2004). The *exoR95* mutant also shows a 70 % reduction in its ability to nodulate alfalfa (Yao *et al.*, 2004). A suppressive mutation, *exoS**, of the *exoR95* mutation was isolated from a pink nodule on alfalfa plants inoculated with the *exoR95* mutant and was mapped genetically to the *S. meliloti* genomic region containing the *exoS* gene (Ozga *et al.*, 1994). The presence of the *exoS** mutation suppressed the succinoglycan-overproduction phenotype of the *exoR95* mutant (Ozga *et al.*, 1994),

The *S. meliloti exoS* gene, which encodes the ExoS sensor with the periplasmic sensing domain and cytoplasmic kinase domain of the ExoS/ChvI two-component regulatory system, was discovered with the isolation of the *exoS96*::Tn*5* mutant (Cheng & Walker, 1998a; Doherty *et al.*, 1988; Osteras *et al.*, 1995). The mutant ExoS96 protein resulting from the Tn*5* insertion appeared to have lost a large part of its first transmembrane domain and to

have become a constitutively active sensor, leading to continuous activation or suppression of ExoS/ChvI-regulated genes (Cheng & Walker, 1998a). These changes were also reflected in succinoglycan overproduction and loss of flagella in the *exoS96* mutant (Yao *et al.*, 2004). Interestingly, the *exoS96* mutation showed little effect on symbiosis (Yao *et al.*, 2004). Efforts to delete the *exoS* genes from the *S. meliloti* genome were unsuccessful until recently, with the use of a merodiploid-facilitated strategy: the complete loss of ExoS affected the growth of *S. meliloti* cells on 21 different carbon sources (Belanger *et al.*, 2009). This is consistent with other findings showing that the ExoS/ChvI system regulates the expression of hundreds of *S. meliloti* genes (Chen *et al.*, 2009; Wang *et al.*, 2010; Wells *et al.*, 2007).

Recent biochemical and genetic analyses of both ExoR and the ExoS/ChvI system have placed them into one signal-transduction pathway (Chen *et al.*, 2008; Wells *et al.*, 2007). ExoR is exported into the periplasm, losing its signal peptide in the process, so that the ExoR protein can be found in two different forms, ExoRp, the full-length precursor form, and ExoRm, the mature form without its signal peptide (Chen *et al.*, 2008). The ExoRm protein may interact directly with the ExoS protein to form an ExoRm–ExoS protein complex, which keeps ExoS in the off state (Chen *et al.*, 2008). Thus, the current hypothesis is that the amount of ExoRm protein in the periplasm modulates the status of ExoS, enabling ExoR to indirectly modulate the expression of hundreds of *S. meliloti* genes regulated by the ExoS/ChvI two-component system. This hypothesis led us to focus on mechanisms regulating *exoR* gene expression.

In this work, we characterized *exoR* expression in different *exoR* and *exoS* genetic backgrounds using an *exoR* promoter–*gfp* fusion. We were able to uncover the regulatory mechanism of *exoR* expression and its effect on *S. meliloti* cells' ability to regulate the expression of a large number of genes regulated by the ExoS/ChvI two-component regulatory system, including succinoglycan and flagellum-biosynthesis genes.

METHODS

Bacterial strains and growth media. The bacterial strains and plasmids used in this study are listed in Table 1. *Escherichia coli* strains were grown in Luria–Bertani (LB) medium at 37 °C (Sambrook *et al.*, 1989). *S. meliloti* was grown in LB medium supplemented with 2.5 mM $MgSO_4$ and 2.5 mM $CaCl_2$ (LB/MC) at 30 °C (Leigh *et al.*, 1985). When required, IPTG was added to induce gene expression at a

Table 1. Strains and plasmids

Strain or plasmid	Relevant properties	References
E. coli		
DH5α	General-purpose strain	Hanahan (1983)
MT616	MT607, pRK600, Cm^R	Finan *et al.* (1986)
S. meliloti		
Rm1021	SU47, Sm^R	
Rm7095	Rm1021 *exoR95*::Tn*5*, Nm^R	Doherty *et al.* (1988)
Rm7096	Rm1021 *exoS96*::Tn*5*, Nm^R	
SmHC20	*exoR95exoS**, Nm^R	This work
SmHC21	*exoR95exoS*$_{supA}$, Nm^R	This work
Plasmids		
pMB393	Cloning vector, Sp^R	Gage *et al.* (1996)
pHC77	pMB393 carrying the *exoX–exoY* intergenic region and *exoY*::*gfp* fusion	Cheng & Yao (2004)
pHC505	pMB393 with the fusion of the *exoR* promoter (−20 to −1 region) and the *gfp* gene	This work
pHC501	pMB393 with the fusion of the *exoR* promoter (−662 to −1 region) and the *gfp* gene	This work
pHC514	pP_{exoR}*gfp*, pMB393 with the fusion of the *exoR* promoter (−325 to −1 region) and the *gfp* gene	This work
pHC548	pMB393 with the fusion of the *exoR* promoter (−662 to −353 and −20 to −1 region) and the *gfp* gene	This work
pSW213	Cloning vector, IncP-derived, *lacI*^{Q}, P_{lac}*lacZ*, Tc^R	Mantis & Winans (1993)
pHC530	pP_{lac}*exoR*, pSW213 with the *exoR* gene expressed from the inducible *lac* promoter	This work
pHC556	pP_{lac}*exoR*Δ*sp*, pSW213 with a mutated *exoR* lacking its signal peptide sequence expressed from the inducible *lac* promoter	This work
pHC560	pP_{lac}*exoS*, pSW213 with the *exoS* gene expressed from the inducible *lac* promoter	This work
pRK600	Helper plasmid, Cm^R	Finan *et al.* (1986)
pJK19-1		

final concentration of 0.8 mM. To examine succinoglycan production on solid medium, calcofluor white M2R (CF, Fluorescent Brightener 28, Sigma) was added to a final concentration of 0.02 % (w/v) in LB/MC agar, which was buffered to pH 7.4 with 10 mM HEPES (Leigh *et al.*, 1985). The following antibiotics were used at the concentrations indicated: chloramphenicol (Cm), 10 μg ml^{-1}; neomycin (Nm), 200 μg ml^{-1}; spectinomycin (Sp), 100 μg ml^{-1}; streptomycin (Sm), 500 μg ml^{-1}, and tetracycline (Tc), 10 μg ml^{-1}.

Construction of ExoR-expressing plasmids. An *Xho*I–*Kpn*I DNA fragment containing the complete *exoR* ORF was obtained by PCR using *S. meliloti* Rm1021 genomic DNA as the template and two PCR primers: *exoRf-20x* and *exoRr807k* (see Supplementary Table S1, available with the online version of this paper). The PCR product was digested with *Xho*I and *Kpn*I, and ligated with similarly digested vector pSW213 to generate plasmid pHC530 (labelled $pP_{lac}exoR$), which expresses the wild-type *exoR* gene from an IPTG-inducible *lac* promoter. Similarly, an *Xho*I/*Kpn*I DNA fragment containing part of the *exoR* gene without the signal-peptide-coding region was obtained by PCR using *S. meliloti* Rm1021 genomic DNA as the template and two PCR primers: *exoRf91x* and *exoRr807k* (Supplementary Table S1). This mutated *exoR*, *exoRΔsp*, was cloned into vector pSW213, giving plasmid pHC556 (labelled $pP_{lac}exoR\Delta sp$) expressing *exoRΔsp* under the control of the same IPTG-inducible *lac* promoter. The ExoRΔsp should have one extra N-terminal methionine compared with ExoRm.

Construction of an ExoS-expressing plasmid. A *Bam*HI–*Kpn*I DNA fragment containing the complete *exoS* ORF (1788 bp) was obtained by PCR using *S. meliloti* Rm1021 genomic DNA as the template and two PCR primers: *exoSf1-atgb* and *exoSr1818k* (Supplementary Table S1). The forward primer *exoSf1-atgb* introduced ATG as the *exoS* start codon, replacing the original TTG (predicted) in the genome. The PCR product was digested with *Bam*HI and *Kpn*I, and ligated with similarly treated vector pSW213 to generate plasmid pHC560 (labelled $pP_{lac}exoS$), expressing the *exoS* gene from the IPTG-inducible *lac* promoter on the vector.

Determination of the *exoS* and *chvI* gene sequences. The ORFs of *exoS* and *chvI* were amplified by PCR using primers listed in Supplementary Table S1 and sequenced at Albert Einstein College of Medicine using primers listed in Supplementary Table S2.

Construction of *exoR* promoter–*gfp* fusions. DNA fragments covering different *exoR* promoter regions were amplified from genomic DNA of *S. meliloti* Rm1021. The DNA fragment containing the *gfp* gene was amplified from plasmid pJK19-1. Primers used are listed in Supplementary Table S1. DNA fragments containing *exoR* promoter–*gfp* fusions (P_{exoR}–*gfp*) were constructed either by joining the *exoR* promoter fragment with the *gfp* gene fragment at a common *Nhe*I site or by recombinant PCR. Each of the P_{exoR}–*gfp* fusions was digested with *Hin*dIII and *Xho*I, and ligated with similarly digested vector pMB393. This set of P_{exoR}–*gfp* fusions, covering the *exoR* gene upstream regions of −20 to −1, −662 to −1, −325 to −1, and −20 to −1 fused with −662 to −353, was expressed from plasmids pHC505, pHC501, pHC514 and pHC548, respectively (Table 1, Fig. 1a). All plasmids were moved into *S. meliloti* strains through conjugation using MT616 as the helper.

Measurement of the *exo* gene promoter activities. GFP fluorescence intensity was used to represent the *exoR* promoter activities in *S. meliloti* cells expressing the P_{exoR}–*gfp* fusions or the *exoY* promoter activities in cells expressing the P_{exoY}–*gfp* fusions as previously described (Cheng & Yao, 2004). Briefly, bacterial cultures were collected, washed, and resuspended in 0.85 % sterile NaCl solution to OD_{600} of about 0.1. Equal volumes of the diluted cultures (100 μl) were transferred to wells of a black 96-well microplate and a transparent 96-well microplate. The cultures in the black microplate were used to determine the intensities of GFP fluorescence using a fluorescence microplate reader (SpectraMax Gemini XS, Molecular Devices). The cultures in the transparent microplate were used to determine cell densities (OD_{600}) using an absorbance microplate reader (SpectraMax 340PC, Molecular Devices). The intensity of GFP fluorescence of each culture was normalized to its corresponding cell density and used to represent the *exoR* or the *exoY* promoter activities.

Alfalfa nodulation assays. Alfalfa nodulation was carried out on plates as previously described (Leigh *et al.*, 1985). Briefly, alfalfa seeds were surface-sterilized in 50 % (v/v) freshly diluted bleach for 10 min,

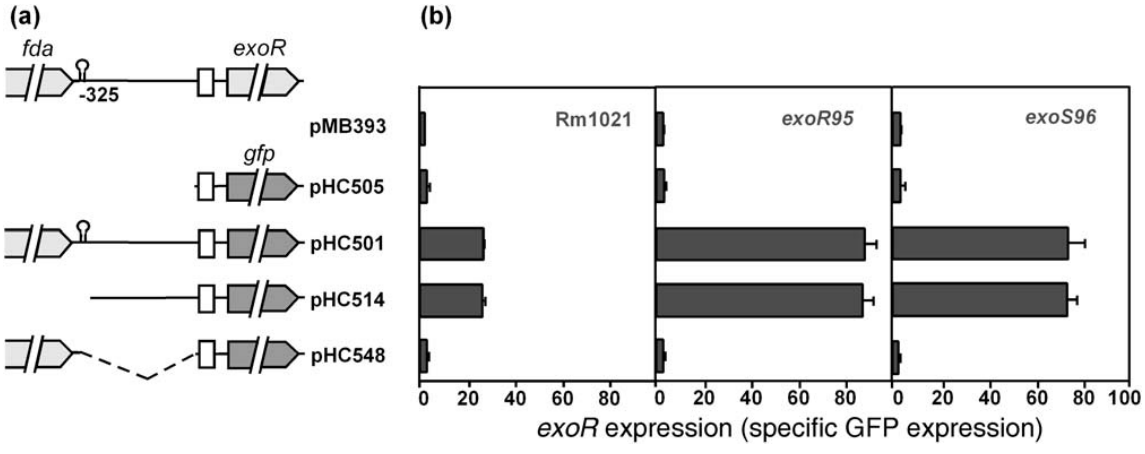


Fig. 1. (a) Schematic representation of the *exoR* gene region in *S. meliloti*. Solid bars indicate the *exoR* promoter regions in the constructs and the dashed line represents the region that was not included. The box indicates the RBS and the hairpin indicates the transcription terminator. (b) *exoR* promoter activities of different constructs in the wild-type Rm1021 and in *exoR95* and *exoS96* mutants. Specific GFP expression was determined by normalizing GFP fluorescence intensity to cell density (OD_{600}) and used to represent *exoR* promoter activity. Data are means and ranges from two independent experiments.

washed in sterile distilled water four times, spread on 0.8 % (w/v) agar, wrapped in aluminium foil, and placed in a plant growth chamber (26 °C) for 40 h for germination. A set of seven seedlings (each about 2.5 cm long) was placed on Jensen's nitrogen-free agar medium in square Petri dishes. *S. meliloti* cells were collected from overnight cultures in LB/MC medium, washed, and diluted with sterile distilled water to OD_{600} 0.03. Cell suspension (1 ml) was spread evenly over the seedlings in each square Petri dish. The Petri dishes were left standing for a few hours to absorb the liquid and then wrapped with aluminium foil on three sides to cover the roots. The Petri dishes with alfalfa plants were placed in the plant growth chamber for 4 weeks. The number of nodules and nodule colour were examined to determine overall symbiosis efficiency.

Isolation of *exoR* suppressor mutation. The suppressor mutation of the *exoR95* mutation was isolated as described previously (Ozga *et al.*, 1994). Briefly, nodules were removed from alfalfa inoculated with the *exoR95* mutant, surface-sterilized by 2 min incubation in 50 % (v/v) Clorox bleach, washed six times in sterile distilled water, and crushed in 100 µl LB/MC with 5.4 % (w/v) glucose inside the wells of a 96-well microplate. The suspensions were diluted 1 : 100 in the same medium and plated on LB/MC/CF agar plates with appropriate antibiotics. Dim colonies, which indicated a reduction in succinoglycan production, were further characterized as having the suppressor mutation for the *exoR95* mutation.

Cell motility. Cell motility was examined using swimming plates as described previously (Yao *et al.*, 2004). Briefly, fresh bacterial cultures were prepared, diluted to an OD_{600} of 0.1, and 2 µl aliquots were pipetted onto LB/MC soft agar (0.3 %) plates and incubated for 3 days.

RESULTS

Analysis of the *exoR* promoter region

To analyse *exoR* expression, a set of fusions of the *gfp* gene to different *exoR* promoter regions was constructed (Fig. 1a). Levels of *exoR* expression were first examined in the wild-type Rm1021 background using specific GFP fluorescence (Fig. 1b), which was generated by normalizing GFP fluorescence intensities to the optical densities of the cultures. A background level of specific GFP fluorescence of 1.99 ± 0.17 was determined using Rm1021 with the expression vector pMB393 without *gfp* (Fig. 1b). Levels of *exoR* expression from the region containing the putative ribosome-binding site (RBS) in plasmid pHC505 and the region upstream of the putative terminator in plasmid pHC548 were 2.81 ± 1.38 and 2.94 ± 0.76, respectively. Levels of *exoR* expression from the −1 to −662 (pHC501) and −1 to −325 (pHC514) regions upstream of the *exoR* gene were 26.95 ± 0.41 and 26.35 ± 1.33, respectively. The latter plasmid, named $pP_{exoR}gfp$, was used to measure *exoR* expression throughout the study. Taken together, these data suggest that the *exoR* promoter is located within the −17 to −325 region upstream of the *exoR* gene, hereafter referred to as the *exoR* promoter region.

exoR expression is upregulated in the *exoR95* and *exoS96* mutant backgrounds

Levels of *exoR* expression from different fusions were also determined in *exoR95* and *exoS96* mutants (Fig. 1b). The *exoR95* and *exoS96* mutants carrying the putative RBS (pHC505) or the region upstream of the putative terminator (pHC548) showed no *exoR* expression. Interestingly, both *exoR95* and *exoS96* mutants carrying the *exoR* promoter region in plasmid $pP_{exoR}gfp$ showed significantly higher levels of *exoR* expression: 87.56 ± 4.40 and 74.07 ± 3.88, respectively. Both *exoR95* and *exoS96* mutants carrying the *exoR* promoter region and the region upstream of the putative terminator in pHC501 showed similarly upregulated levels of *exoR* expression: 88.32 ± 4.92 and 74.38 ± 7.32, respectively. Compared with Rm1021 carrying the same plasmids, these data clearly suggest that *exoR* expression is upregulated about threefold in the *exoR95* and *exoS96* mutants, raising the possibility that the ExoS/ChvI two-component regulatory system is involved in regulating *exoR* expression.

The upregulation of *exoR* expression was compared with that of the *exoY* gene, which is the best-known regulatory target of the ExoS/ChvI system. *exoY* expression was measured using a fusion of the *gfp* gene to the *exoY* promoter and part of the *exoY* gene on plasmid pHC77 ($pP_{exoY}gfp$). The levels of *exoY* expression in the *exoR95* mutant was increased sixfold compared with the levels in the wild-type Rm1021 strain while the expression of *exoR* was increased threefold in the side-by-side comparison (Fig. 2). These results suggest that *exoR* expression is upregulated in the absence of functional ExoR protein but the level of upregulation is less than that of *exoY*.

Characterization of two *exoS* mutations

To examine the possible involvement of the ExoS protein in regulating *exoR* expression, two *exoS* mutations, *exoS** and $exoS_{supA}$, were further characterized. The *exoS** mutation has been isolated and mapped genetically (Ozga *et al.*, 1994). The $exoS_{supA}$ mutation was isolated recently in our lab using the same approach as that used to isolated the *exoS** mutation.

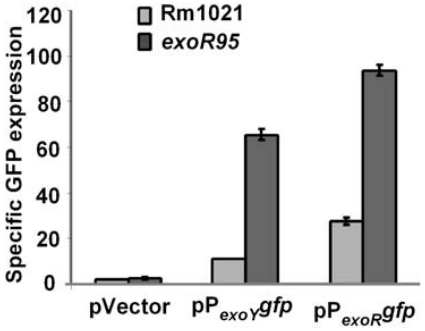


Fig. 2. Comparison of *exoY* and *exoR* promoter activities in wild-type Rm1021 and the *exoR95* mutant using fusion of the *gfp* gene to the *exoY* promoter on plasmid pHC77/ pP_{exoY} *gfp* and to the *exoR* promoter on pHC514/pP_{exoR} *gfp*. The results are means ± SE of three independent experiments.

The presence of either the *exoS** or the $exoS_{supA}$ mutation suppressed the phenotypes of the *exoR95* mutant, so that both *exoR95exoS** and *exoR95*$exoS_{supA}$ double mutants showed wild-type levels of succinoglycan, motility (Fig. 3) and symbiosis with alfalfa (Table 2).

Our sequencing results showed that the *exoS** mutation is a single A-to-G base substitution resulting in a threonine-to-alanine (T150A) change at position 150 of ExoS (Fig. 4). The $exoS_{supA}$ mutation is a single C-to-T base substitution resulting in an alanine-to-valine (A76V) change at position 76 of ExoS (Fig. 4). Both *exoS** and $exoS_{supA}$ mutations are located in the sensing domain of ExoS, which suggests that these mutations alter the sensing function or status of the ExoS protein to a constant low level, resulting in suppression of succinoglycan overproduction and of other phenotypes of the *exoR95* mutant.

Upregulation of *exoR* expression is blocked by *exoS** and $exoS_{supA}$ mutations

The effects of the *exoS** and $exoS_{supA}$ mutations on the upregulation of *exoR* expression observed in mutants *exoR95* and *exoS96* were further studied to determine whether the ExoS/ChvI two-component regulatory system is involved in regulating *exoR* expression. Levels of *exoR* expression in different strains were determined using the *exoR* promoter–*gfp* fusion on plasmid $pP_{exoR}gfp$. Levels of *exoR* expression in *exoR95exoS**($pP_{exoR}gfp$) and *exoR95*$exoS_{supA}$ ($pP_{exoR}gfp$) mutants were 32.26 ± 2.59 and 32.57 ± 3.48, respectively, similar to that of Rm1021($pP_{exoR}gfp$) (Fig. 5). These data suggested that both *exoS** and $exoS_{supA}$ mutations block the *exoR95*-induced upregulation of *exoR* expression.

To further confirm this finding, both *exoS** and $exoS_{supA}$ mutations were complemented with wild-type *exoS* carried on a compatible plasmid, $pP_{lac}exoS$. The presence of extra copies of *exoS* showed no apparent effect on *exoR* expression in wild-type Rm1021, further elevated *exoR* expression in the *exoR95* mutant background, and decreased *exoR* expression in the *exoS96* mutant to wild-type levels (Figs 3 and 5), which is consistent with the *exoS96* mutation being recessive (Cheng & Walker, 1998a). Most importantly, ExoS brought levels of *exoR* expression in *exoR95exoS**($pP_{exoR}gfp$, $pP_{lac}exoS$) and *exoR95*$exoS_{supA}$($pP_{exoR}gfp$, $pP_{lac}exoS$) close to the level of *exoR* expression in the *exoR95*($pP_{exoR}gfp$) single mutant (87.56 ± 4.40). Taken together, these data show that levels of *exoR* expression are clearly affected by the functional status of the ExoS protein, suggesting that *exoR* expression is most likely regulated through the ExoS/ChvI two-component regulatory system.

exoR expression is only autoregulated by full-length ExoR protein

The loss of functional ExoR protein in the *exoR95* mutant led to upregulation of *exoR* expression. This finding and previous reports of ExoR–ExoS interactions (Chen *et al.*, 2008) raised the possibility that ExoR is involved in regulating its own expression through an interaction with the ExoS sensor in the periplasm. To test this, a mutated form of ExoR without its conserved signal peptide, ExoRΔSP, was constructed and expressed from plasmid $pP_{lac}exoR\Delta sp$, which is compatible with plasmid $pP_{exoR}gfp$. We have previously found that the ExoRΔSP protein remains in the cytoplasm (unpublished) so it should not be able to interact with the periplasmic ExoS sensing domain. When both ExoR and ExoRΔSP were expressed in Rm1021, and in *exoR95*, *exoS96*, *exoR95exoS** and *exoR95*$exoS_{supA}$ mutants, the only significant difference in *exoR* expression was found in the *exoR95* mutant (Fig. 6). These data suggest that the wild-type ExoR, but not ExoRΔSP, is able to suppress *exoR* gene expression. Taken together, these

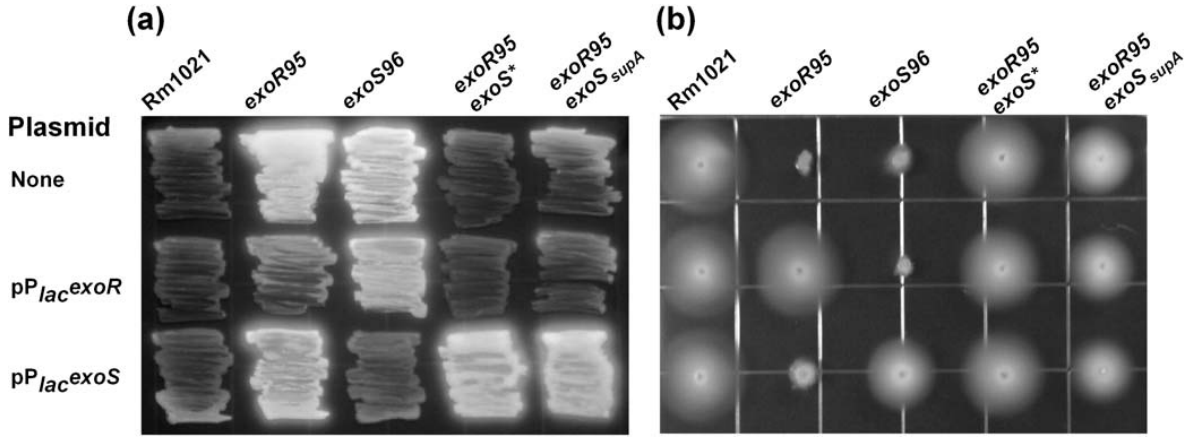


Fig. 3. Abilities of *S. meliloti* cells to produce succinoglycan (a) and to swim (b) were examined on plates containing LB/MC/CF/IPTG medium and LB/MC/IPTG with 0.3% agar, respectively. Intensity of CF fluorescence represents level of succinoglycan production (a). The *exoR* gene was expressed from the *lac* promoter on plasmid $pP_{lac}exoR$/pHC530. The *exoS* gene was expressed from the *lac* promoter on plasmid $pP_{lac}exoS$ /pHC560.

Table 2. Nodulation efficiency of different *S. meliloti* strains

Percentage of pink nodules and average number of nodules per plant were determined using seven 4-week-old alfalfa plants for each of the six bacterial strains. The results are means ± SE of three independent repeats.

Strain	Pink nodules (%)	Nodules per plant
Rm1021	94.15 ± 5.58	4.79 ± 0.38
exoY210	0.00 ± 0.00	5.43 ± 0.65
exoR95	15.74 ± 4.03	7.00 ± 0.65
exoS96	79.33 ± 6.51	5.93 ± 1.17
*exoR95exoS**	95.96 ± 7.00	5.43 ± 1.08
exoR95exoS$_{supA}$	95.63 ± 5.12	4.43 ± 0.76

results imply that ExoR regulates its own expression through the ExoS/ChvI two-component regulatory system.

Kinetic analysis of ExoR autoregulation

To examine ExoR autoregulation in real time, the *exoR* gene expressed from the *E. coli lac* promoter on plasmid pP$_{lac}$*exoR* was used to examine the link between the amount of intracellular ExoR protein and levels of *exoR* expression. The *exoR* promoter–*gfp* fusion on plasmid pP$_{exoR}$*gfp* was used to monitor levels of *exoR* expression. Overnight cultures of Rm1021(pP$_{exoR}$*gfp*), Rm1021(pP$_{exoR}$*gfp*, pP$_{lac}$*exoR*), *exoR95*(pP$_{exoR}$*gfp*) and *exoR95*(pP$_{exoR}$*gfp*, pP$_{lac}$*exoR*) were prepared in the presence of IPTG to ensure high levels of intracellular ExoR. Cells from half of each culture were washed and resuspended in the same medium without IPTG. Levels of *exoR* expression were determined at 6, 12 and 24 h after removal of IPTG. *exoR* expression by Rm1021(pP$_{exoR}$*gfp*) and Rm1021(pP$_{exoR}$*gfp*, pP$_{lac}$*exoR*) was not affected by the removal of IPTG (Fig. 7a). *exoR* expression remained at high levels in the *exoR95*(pP$_{exoR}$*gfp*) mutant with or without IPTG (Fig. 7b); it remained at low levels in the *exoR95*(pP$_{exoR}$*gfp*, pP$_{lac}$*exoR*) mutant in the continuous presence of IPTG, and in the *exoR95*(pP$_{exoR}$*gfp*, pP$_{lac}$*exoR*) mutant it increased after the removal of IPTG (Fig. 7b). This latter increase in *exoR* expression was detectable 6 h after IPTG removal and expression reached

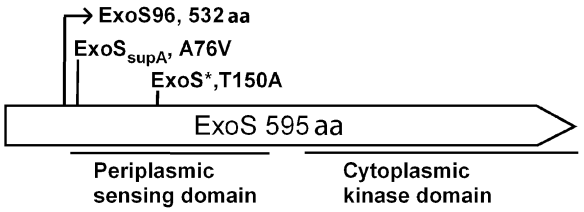


Fig. 4. Schematic diagram of ExoS protein showing the periplasmic sensing and cytoplasmic kinase domains. The starting position of the ExoS96 mutant protein (532 aa), and the positions of two spontaneous mutations, *exoS** and *exoS*$_{supA}$, are indicated.

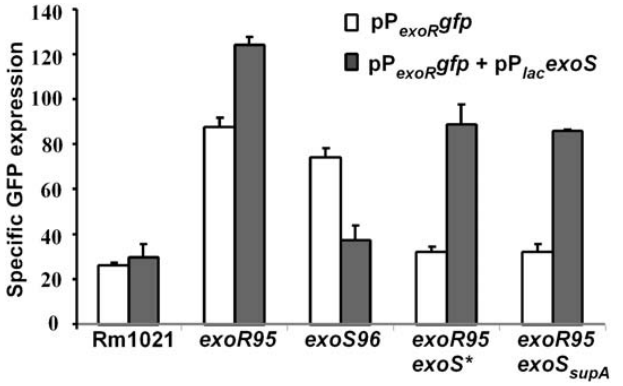


Fig. 5. *exoR* expression in different genetic backgrounds with or without the presence of the wild-type *exoS* gene. The *exoR* promoter was fused to the *gfp* gene and expressed from plasmid pP$_{exoR}$*gfp* (pHC514). The wild-type *exoS* gene was expressed using the *E. coli lac* promoter from plasmid pP$_{lac}$*exoS* (pHC560). Data represent means ± ranges from two independent experiments.

a level below that of the *exoR95*(pP$_{exoR}$*gfp*) after 24 h (Fig. 7b). This could be because *exoR95*(pP$_{exoR}$*gfp*, pP$_{lac}$*exoR*) had a low level of ExoR protein expressed from the leaky *lac* promoter, which cannot be completely shut off, even in the presence of LacI^{Q} expressed from the same pP$_{lac}$*exoR* plasmid. These data showed that a reduction in the amount of ExoR protein results in increased *exoR* expression, providing real-time evidence of ExoR's negative regulation of its own expression.

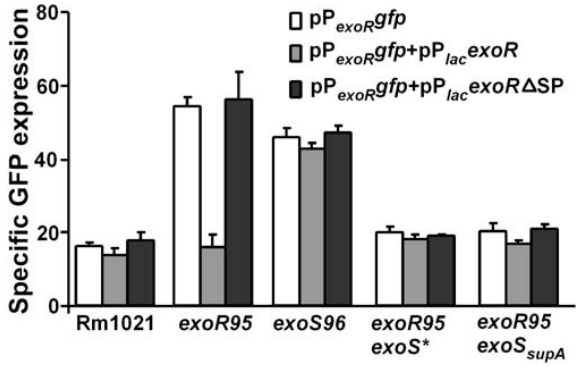


Fig. 6. *exoR* expression in different genetic backgrounds with or without the wild-type ExoR and mutated ExoR without its native signal peptide (ΔSP). The *exoR* promoter–*gfp* fusion, P$_{exoR}$*gfp*, was expressed from plasmid pP$_{exoR}$*gfp* (pHC514) to monitor levels of *exoR* expression. The wild-type ExoR protein was expressed from the *lac* promoter on plasmid pP$_{lac}$*exoR* (pHC530) and the mutated ExoRΔSP was expressed from the *lac* promoter on plasmid pP$_{lac}$*exoR*Δ*sp* (pHC556). Data represent means ± ranges from two independent experiments.

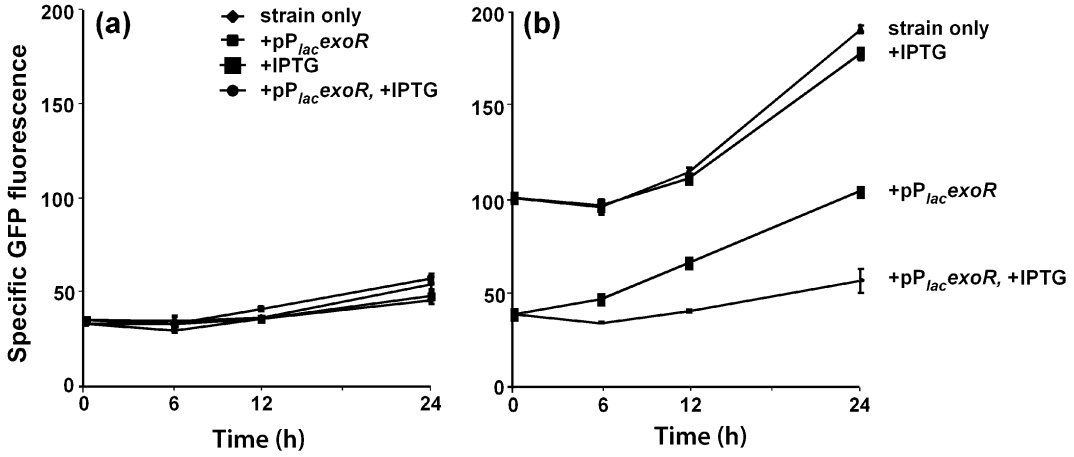


Fig. 7. Effects of total ExoR protein reduction on *exoR* expression in wild-type Rm1021(pP$_{exoR}$*gfp*) (a) and in the *exoR95*(pP$_{exoR}$*gfp*) mutant background (b) with or without IPTG induction. *exoR* expression was monitored using a fusion of the *exoR* promoter and *gfp*, expressed from plasmid pP$_{exoR}$*gfp* (pHC514). The amount of total ExoR protein was regulated using a fusion of the *lacZ* promoter and the *exoR* gene, expressed from plasmid pP$_{lac}$*exoR* (pHC530). The curves are labelled for the strain alone or with additional plasmid pP$_{lac}$*exoR* or IPTG or both. Data represent means ± ranges from two independent experiments.

DISCUSSION

The periplasmic *S. meliloti* ExoR protein and ExoS/ChvI two-component regulatory system regulates the production of succinoglycan and flagella (Yao *et al.*, 2004). ExoR is a negative regulator of the ExoS sensor (Chen *et al.*, 2008). This would suggest that the amount of ExoR protein can affect the expression of hundreds of ExoS/ChvI-system-regulated genes. Thus, we focused on the regulation of *exoR* expression, which could lead to the discovery of the key factor(s) or regulator(s) functioning upstream of ExoR in this essential ExoR–ExoS/ChvI regulatory cascade.

To monitor *exoR* expression and identify its potential regulators, the maximum region upstream of the *exoR* gene which contains *exoR* promoter activities was identified using a set of nested deletions in the region. Interestingly, activities of the *exoR* promoter showed about a threefold increase in both *exoR95* and *exoS96* mutants. This threefold increase of *exoR* expression is smaller than the sixfold increase of the *exoY* gene in the same loss of ExoR function *exoR95* mutant. This raised the possibility that *exoR* expression may be regulated by the ExoS/ChvI system directly or indirectly and that ExoR protein might be involved in regulating its own expression.

To directly characterize the link between the amount of total ExoR protein and *exoR* expression level, total ExoR production was regulated using an inducible *E. coli lac* promoter on plasmid pP$_{lac}$*exoR*, which also expresses the LacI^{Q} protein. Suppression of the *lac* promoter by LacI^{Q} is not complete, which means that a small amount of total ExoR protein will be expressed from the *lac* promoter in the absence of inducer. We found that upregulation of *exoR* expression in the *exoR95* mutant could be suppressed by the presence of plasmid pP$_{lac}$*exoR*, with or without IPTG, suggesting that the presence of even small amounts of total ExoR can suppress *exoR* expression. In addition, when the amount of total ExoR was artificially reduced by removing IPTG, the level of *exoR* expression increased and stabilized at a new level, which was between those in the *exoR95* mutant and in the wild-type Rm1021. These findings support the notion that higher levels of total ExoR protein will result in lower levels of *exoR* expression, suggesting that ExoR negatively regulates its own expression.

Because ExoR is the negative regulator of the ExoS sensor of the ExoS/ChvI system, ExoS could be involved in mediating ExoR autoregulation. Our initial finding of upregulation of *exoR* expression by the *exoS96* mutant supports this possibility. In addition, we found that a mutated ExoR lacking its signal peptide, which could not be exported to the periplasm, was unable to affect *exoR* expression. Although other possibilities exist, these findings raise the possibility that ExoR can only autoregulate in the periplasm, via the ExoS/ChvI system.

If the ExoS/ChvI system is indeed involved in regulating *exoR* expression, mutations in the *exoS* gene should block the effects of ExoR protein amount on *exoR* expression. We analysed two spontaneous *exoS* mutations, *exoS** and *exoS*$_{supA}$, that suppress succinoglycan overproduction, as well as other phenotypes of the *exoR95* mutant. Both mutations are located in the ExoS periplasmic sensing domain and they may alter the structure of ExoS such that it remains in the inactive state in the absence of functional ExoR suppressor. Our data clearly show that the presence of either one of these two mutations suppresses upregulation of *exoR* expression in the *exoR95* mutant background.

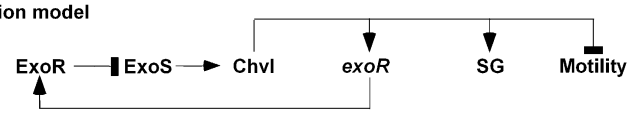
The suppressive effects of *exoS** and $exoS_{supA}$ mutations on *exoR95* succinoglycan overproduction were reversed by the presence of the wild-type *exoS* gene, which strongly suggest that the ExoS/ChvI system mediates ExoR autoregulation of *exoR* expression. Interestingly, the suppressive effects of *exoS** and $exoS_{supA}$ mutations on *exoR95* motility were not reversed by the presence of wild-type *exoS* (Fig. 3). One possible explanation is that the activation of *exo* expression and the suppression of *fla* expression may require different levels of ExoS/ChvI activation. The ExoS sensor probably functions in dimeric form, so the *exoR95exoS**(pP_{lac}*exoS*) mutant should have ExoS*/ExoS*, ExoS/ExoS* and ExoS/ExoS dimers. Both ExoS*/ExoS* and ExoS/ExoS* dimers might not be active due to the presence of ExoS*, so the *exoR95exoS**(pP_{lac}*exoS*) mutant would have a small amount of ExoS/ExoS dimers. This could activate the ExoS/ChvI system enough to upregulate *exo* expression but not enough to suppress *fla* expression. The $exoS_{supA}$ mutation may function in the same way. Both *exoR95exoS**(pP_{lac}*exoS*) and *exoR95*$exoS_{supA}$(pP_{lac}*exoS*) mutants will be analysed in the future to further confirm the presence and the biological significance of such differential regulation.

A model of ExoR autoregulation through the ExoS/ChvI system is proposed based on our new findings and previous understanding of ExoR and the ExoS/ChvI system (Fig. 8), suggesting the way in which total ExoR protein is regulated and how levels of total ExoR protein, especially mature ExoRm, in the periplasm can modulate all of the genes regulated by the ExoR–ExoS/ChvI pathway. In a wild-type Rm1021 cell, reduction in ExoRm will lead to activation of ExoS, direct or indirect upregulation of *exoR* expression, accumulation of ExoRp and ExoRm protein, and consequent suppression of ExoS. On the other hand, accumulation of ExoRm in the Rm1021 periplasm will lead to suppression of ExoS, suppression of *exoR* expression, reduction of ExoRm protein, and consequent activation of ExoS. This proposed system would enable *S. meliloti* cells to maintain a stable level of *exoR* expression based on the levels of total ExoR protein. Any disruptions in the pathway, such as loss of functional ExoR protein or constitutively active ExoS sensor, will disrupt ExoR regulation. As demonstrated here, the effects of such disruptions can be blocked by suppressor mutations in the *exoS* gene. Altogether, our new findings suggest that ExoR autoregulation may play a key role in regulating the activity levels of ExoS sensor.

ExoR autoregulation would also make it possible for *S. meliloti* cells to maintain the expression of a large number of ExoS/ChvI-regulated genes at relatively constant levels, and to return expression to those levels after any changes in ExoS activity. This would enable the bacterial cells to respond to the appearance of environmental stimuli by altering the expression of relevant genes, and quickly return their expression to 'normal' levels after disappearance of those stimuli. This might be the mechanism that allows *S. meliloti* cells to produce succinoglycan once they are trapped inside curled alfalfa root hairs and to terminate succinoglycan production upon entering the alfalfa root nodules. Loss of ExoR in the *exoR95* mutant causes the mutant to remain in succinoglycan-overproducing mode and reduces its symbiotic efficiency, which further argues for the biological importance of ExoR autoregulation. We are currently researching ways to improve our ability to detect signals for the ExoS/ChvI system, which will greatly improve our understanding of the signalling between *S. meliloti* and alfalfa during nodulation.

Autoregulation, especially single-gene autoregulation, is quite common in *S. meliloti*, such as MucR autoregulation (Bahlawane *et al.*, 2008), although fewer examples of autoregulation by periplasmic proteins are known. One such example is the *E. coli* periplasmic CpxP protein, which autoregulates through the CpxA/CpxR two-component regulatory system (Dong *et al.*, 1993; Fleischer *et al.*, 2007; Raivio & Silhavy, 1999; Wolfe *et al.*, 2008). The CpxP protein normally forms a protein complex with CpxA, keeping the latter in the off state. Environmental stress, such as changes in pH, triggers the misfolding of periplasmic proteins. These misfolded proteins form complexes with CpxP, which are then degraded by DegP protease in the periplasm (Buelow & Raivio, 2005; Isaac *et al.*, 2005). Thus released from the CpxP–CpxA complex, CpxA is activated and turns on the expression of all CpxA/CpxR-regulated genes, including *cpxP*, leading to CpxP suppression of CpxA (Buelow & Raivio, 2005; Isaac *et al.*,

(a) ExoR autoregulation model



(b) Protein levels or activities

Strain	Predicted	Predicted	Predicted	Observed	Observed	Observed
Rm1021	Low	Low	Low	Low	Low	High
exoR95	Absent	High	High	High	High	Low
exoS96	High	Always On	High	High	High	Low
*exoR95exoS**	Absent	Always Low	Low	Low	Low	High
*exoR95*$exoS_{supA}$	Absent	Always Low	Low	Low	Low	High

Fig. 8. (a) Schematic representation of the ExoR–ExoS/ChvI regulatory pathway and (b) levels of total ExoR protein, *exoR* expression, succinoglycan (SG) production and motility in different *S. meliloti* genetic backgrounds. The status of ExoS and ChvI proteins was predicted based on prior knowledge of the system.

2005). Environmental stress conditions such as osmotic pressure and pH were able to modulate succinoglycan production (Hellweg *et al.*, 2009). While the ExoS/ChvI pathway may be involved in sensing common stress conditions, it is also possible that the system is involved in sensing plant signals. Further study of the ExoR–ExoS/ChvI signalling pathway will provide new insights into bacterial signalling and sensing in microbe–plant interactions.

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